Metal Content of Tobacco Mosaic Virus and Tobacco Mosaic Virus RNA*

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RNA obtained from a wide variety of species contains significant quantities of firmly bound metals. The functional significance of these analytically reproducible compositional findings has been uncertain, and a number of approaches have been employed to discern the possible biological implication of these observations (Wacker, 1962). Since the RNA of viruses has a known biological function, TMV was chosen in an attempt to correlate the metal content with the known infectivity of the RNA from this agent. The data demonstrate that tobacco mosaic virus itself and its component RNA consistently contain magnesium, calcium, strontium, barium, aluminum, chromium, manganese, iron, nickel, zinc, and copper. Metal ions, especially those of the first transition period of the periodic system, are very firmly bound, resisting removal by exhaustive dialysis against metal-free water or chelating agents.

METHODS

The TMV (common strain) used in these studies was prepared by the method outlined by Knight (1962). The virus was grown in Turkish tobacco var. Maryland, Marglobe tomato, or *Physalis floridana*. Only virus preparations which gave clear, colorless pellets upon centrifugation were used. Every effort was made to avoid the introduction of extraneous metal ions. All metal parts of equipment coming in contact with the virus (such as centrifuge tube caps) were coated with silicone grease. TMV was dialyzed exhaustively against metal-free water in cellulose tubing rendered metal-free before use (Hughes and Klotz, 1956). For metal analysis, aliquots of virus were transferred from the dialysis tubing to tared platinum dishes and dried at 104° until a minimum weight was reached. The samples were ashed at 450° in a quartz-lined muffle furnace, then analyzed for metals with a spark excitation system and porous cup electrode as previously described (Vallee, 1955).

TMV-RNA prepared according to the procedure of Gierer and Schramm (1956) was dialyzed against metal-free water, weighed, ashed, and analyzed in the same manner as TMV. Copper was measured spectrophotometrically by means of a method employing sodium diethyldithiocarbonate (Gubler *et al.*, 1952). Glassware and platinum were rendered free of metal contamination as previously described (Thiers, 1957). Reagent-grade chemicals were used throughout. Phenol was redistilled before use. The infectivity of the virus and virus nucleic acid was determined according to established procedures (Fraenkel-Conrat, 1959).

RESULTS

Table I demonstrates four separate analyses performed on aliquots of a single preparation of TMV to test the suitability and repeatability of the spectrographic method for these studies. The metal content of seven different preparations of TMV is shown in Table II.[1] A total of twenty-six individual analyses were performed on seven separate preparations of tobacco mosaic virus. The data represent the mean ± the standard deviation. In addition to the metals listed in Table II, traces of lead were present in two of the seven preparations. All preparations contained strontium and barium in amounts well below 0.5 and 0.1 gram atom per mole, respectively. The metal content of the virus grown on two alternate hosts, tomato and *Physalis floridana*, was also determined. Table III compares the metal content of virus obtained from tobacco plants with that obtained from the other hosts. There is no significant difference in metal content between these viruses.

The virus was dialyzed three times against a 10-fold volume of 0.1 M EDTA at *p*H 7.0 in order to determine the firmness of binding of metals to TMV. EDTA was then removed by dialysis against metal-free water, and the TMV was analyzed spectrographically. Table IV compares the metal content of the virus prior to dialysis with that observed after dialysis. The infectivity of the virus was not altered by this treatment.

TMV-RNA was analyzed spectrographically in similar fashion; the metal content is shown in Table V. These data represent the mean of eleven analyses of four separate preparations of TMV-RNA. All metals listed were detected in each sample. The metal content of TMV-RNA obtained from virus grown on tomato plants and *P. floridana* did not differ significantly from that of RNA obtained from virus grown on tobacco plants.

Samples were heated in the presence of EDTA and reprecipitated with redistilled ethanol in an attempt to reduce the intrinsic metal content of TMV-RNA. Exposure to heat was quite brief, samples being maintained at 65° for 5 minutes, then cooled quickly. A sample of RNA was divided into four portions. The "control" was unheated, and the "heated control" raised to 65° for 5 minutes in 0.025 M ammonium acetate–sodium chloride buffer, *p*H 6.8. The "EDTA control" was made 0.01 M with respect to EDTA, and the "EDTA-heated" sample was in addition heated for 5 minutes at 65°. All samples were then dialyzed against metal-free water and analyzed spectrographically. The metal content of these samples is

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[1] Virus samples were also prepared by the method of Simmons (personal communication). Since the metal content of the virus prepared by this procedure was somewhat higher than that of virus prepared using Knight's procedure and the yields were much lower, no further use was made of this procedure.

TABLE I
METAL CONTENT OF TOBACCO MOSAIC VIRUS
(μg metal/g dry wt)

Sample	Mg	Ca	Al	Cr	Mn	Fe	Ni	Zn	Sr	Ba
1	20	80	15	0.9	0.2	17	8.7	23	1.8	3.5
2	20	90	15	0.9	0.2	20	3.0	27	1.8	4.4
3	13	100	22	1.1	0.4	23	7.2	23	2.4	6.2
4	17	90	20	0.9	0.4	17	7.2	24	2.2	6.1

Host = Tobacco

TABLE II
METAL CONTENT OF TOBACCO MOSAIC VIRUS OBTAINED FROM TURKISH TOBACCO VAR. MARYLAND

Mean ± the standard deviation of 27 analyses performed on seven preparations in gram atoms of metal/mole of virus.

Metal	Mean
Mg	26 ± 9
Ca	150 ± 59
Al	36 ± 18
Cr	0.8 ± 0.25
Mn	0.6 ± 0.24
Fe	12 ± 3
Ni	5 ± 2
Zn	25 ± 13

TABLE III
METAL CONTENT OF TMV OBTAINED FROM THREE DIFFERENT HOSTS

Results are given in gram atoms of metal/mole of virus.

Metal	Tobacco	Tomato[a]	*Physalis floridana*[b]
Mg	26	86	54
Ca	150	200	145
Al	36	33	24
Cr	0.8	1.2	1.2
Mn	0.6	1.2	Not detected
Fe	12	15	14
Ni	5	11	7
Zn	25	32	20
Cu		7	

[a] Average of 8 analyses on 2 preparations. [b] Average of 4 analyses on 1 preparation.

TABLE IV
EFFECT OF DIALYSIS WITH 0.1 M EDTA ON THE METAL CONTENT OF TMV OBTAINED FROM TOBACCO PLANTS

Results in gram atoms/mole of virus.

Metal	Before Dialysis	After Dialysis
Mg	26	9.5
Ca	150	20.0
Al	36	6.4
Cr	0.8	1.7
Fe	12	8.0

presented in Table VI. The content of magnesium, iron, and chromium is quite stable. The concentration of these elements is not reduced by heating even in the presence of EDTA. The increase in calcium content of the EDTA control may be attributed to extraneous contamination. The addition of EDTA or brief heating in its presence did not alter biological activity; within experimental limits (± 10%) the infectivity of all samples was the same. A decrease in infectivity of about 5% would be expected under these conditions (Gordon *et al.*, 1963).

TABLE V
METAL CONTENT OF TMV-RNA OBTAINED FROM TOBACCO PLANTS

Mean ± the standard deviation of eleven analyses of four preparations in gram atoms of metal/mole of RNA.[a]

Mg	6.5 ± 2
Ca	33 ± 16
Al	4.4 ± 1.3
Cr	0.5 ± 0.1
Mn	0.2 ± 0.04
Fe	2.2 ± 0.36
Ni	0.5 ± 0.15
Zn	3.1 ± 0.5
Cu[b]	0.9

[a] All preparations contained very small amounts of strontium and barium. [b] Average of duplicate analyses on 1 sample.

TABLE VI
THE EFFECT OF HEAT AND EDTA ON THE METAL CONTENT OF TMV-RNA GROWN ON TOBACCO PLANTS
(μg/g dry weight)

Metal[a]	Control	Heated Control	EDTA Control	EDTA Heated
Mg	24	21	27	24
Ca	39	88	120	59
Fe	48	49	49	48
Cr	9.5	7	5	7

[a] Zinc was present in all samples; estimated concentration = 10–15 μg/g.

DISCUSSION

The metal contents of TMV and its component RNA reported in these studies are similar to those previously reported for the RNA obtained from many different species (Wacker and Vallee, 1959). Moreover, the iron content of the whole virus and the magnesium, calcium, iron, and copper contents of the TMV-RNA are almost identical to those previously reported by Haschemeyer and also to one of those reported by Loring (Haschemeyer *et al.*, 1959; Loring and Waritz, 1957). The data presented here demonstrate clearly that TMV-RNA and TMV contain significant and reproducible amounts of magnesium, calcium, aluminum, chromium, manganese, iron, nickel, zinc, and copper.

Great care was employed to render all TMV preparations free of loosely bound metals prior to analysis by extensive dialysis. The isolation of TMV-RNA was carried out with reagents which were purified and analyzed before use (Wacker and Vallee, 1959). As a check against possible contamination of the virus by metal ions present in the host, virus preparations from three different hosts were analyzed and shown to be similar. The constancy of the metal content of the preparations examined here as well as those studied in other laboratories by somewhat different procedures (Haschemeyer *et al.*, 1959; Loring and Waritz, 1957)

is strong evidence that the metals are an intrinsic component of TMV and TMV-RNA. This interpretation is strengthened by the demonstration that exposure to EDTA does not remove metals from TMV-RNA even at an increased temperature. The metal content of the whole virus is considerably higher than that of the TMV-RNA; e.g., summing up all the metals found, 260 gram atoms of metal per mole of whole virus are found as compared to 50 gram atoms per mole of TMV-RNA. The reduction in the intrinsic metal content as a result of the extraction of RNA from the virus may indicate that the whole virus binds a considerable quantity of metal which resists dialysis against buffers or metal-free water but is removed by dialysis against EDTA. After dialysis against EDTA, a preparation is obtained which has a metal content approaching that of TMV-RNA. This experiment demonstrates that metal is bound to TMV in two different ways: (1) by binding to the protein moiety, and (2) by binding to the RNA so strongly that the metal resists removal by EDTA.

These results on TMV-RNA are similar to those obtained with RNA from a number of other sources. The metals found in beef liver RNA are not removed by extensive dialysis against a variety of chelating agents (Wacker and Vallee, 1959) and have been considered to be bound "intrinsically." Moreover, iron is bound to beef liver RNA so firmly that upon addition of 1,10-phenanthroline a mixed complex consisting of (RNA·Fe) OP_n can be demonstrated spectrophotometrically (Wacker and Vallee, 1959; Wacker and Williams, in preparation).

While the sites of RNA which bind metals have not been identified with certainty, it would appear that the firmness of binding precludes the assumption that a simple complex such as might form with phosphates can be held responsible. Hence, a different ligand group must be involved. Metals, of the first transition group in particular, are known to form complexes with adenine nucleotides in which the metal is bound to the amino nitrogen of C_6 and N_7 of the purine ring. Moreover, while magnesium binds to only the phosphate groups of ATP, zinc and manganese are bound to phosphate and to N_7 of the purine ring (Cohn, 1962). Mercury and silver have also been shown spectrophotometrically to bind to the nitrogenous bases of DNA and RNA (Yamane and Davidson, 1961, 1962a,1962b; Singer and Fraenkel-Conrat, 1962; Katz and Santilli, 1962). It is probable, therefore, that the metals intrinsic to RNA are bound as chelate complexes to the nitrogenous bases. The extreme firmness of binding of the transition metals in RNA has suggested that they may be bound as "sandwich complexes" similar to dicyclopentadienyliron (ferrocene). While "sandwich complexes" between metals and nitrogenous bases have not been demonstrated, the aromatic ring structure of these compounds renders them capable of reacting to form these very stable complexes (Wacker and Vallee, 1959). It should be noted that experiments on enhancement of nuclear relaxation have indicated that Fe^{+++} is bound in the interior of a DNA molecule in such a manner that the Fe^{+++} does not readily react with protons in the surrounding solvent (Eisinger *et al.*, 1961).

These compositional studies of TMV and TMV-RNA were undertaken in an effort to determine precisely the metal content of a nucleoprotein and its component RNA, which has a well-known organic composition and structure and a well-characterized biological function. Data such as those reported here are required as the first step leading to the assessment of the functional importance of the presence of metals in RNA. While no metal other than magnesium participates directly in the currently known *in vitro* systems which incorporate amino acids into proteins, the repetitive demonstration that metals are always present in RNA suggests that they may serve a functional role. It is clear that metals could add further specificity to any code for protein synthesis which is currently postulated to utilize only the sequence of bases. In addition, it should be pointed out that metals of the first transition group of the periodic system, either intrinsic to RNA or added to RNA, stabilize its secondary structure in a manner similar to that produced by disulfide bridges in proteins (Fuwa *et al.*, 1960; Gordon *et al.*, unpublished). Such structural stabilization may well have functional consequences.

Finally, recent studies using the flagellate *Euglena gracilis* grown in a zinc-deficient medium have demonstrated a marked reduction in both RNA and protein content of the deficient organism (Wacker, 1962). These findings demonstrate that zinc is required for normal protein synthesis and suggest further that the presence of metals in RNA has functional significance. A similar approach has been attempted with TMV. Attempts have been made to induce a metal deficiency in the host, obtain virus from the deficient plants, and then correlate the changes in metal content with alterations in virus infectivity, stability, or organic composition. This study has been unsuccessful thus far since it has not been possible to induce a *quantitative* metal deficiency in tobacco plants. While plants grown in limiting amounts of iron show classical botanical symptoms such as marked chlorosis and stunting of growth, the virus obtained from such plants had a normal iron content. It would appear that the iron deficiency limits the growth of the host long before it limits growth of the virus.

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Biosynthesis of Gramicidins and Tyrocidines in Cell-Free Preparations from *Bacillus brevis**

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A cell-free system, prepared from *Bacillus brevis* and consisting of ribosomes plus a 140,000 × *g* supernatant solution, was active in incorporating suitable C^{14}-amino acids into gramicidins and tyrocidines in the presence of magnesium ions, ATP plus an ATP generator, glutathione, and an amino acid mixture. The process proceeded optimally at approximately *p*H 7.5–7.9, and continued at undiminished rate for at least 4 hours. L-Ornithine, D-valine, and D-phenylalanine were among the isotopic amino acids found to be incorporated into the polypeptides. Glutamic acid was better utilized than was glutamine for tyrocidine synthesis. By reisolating the ribosomes at the end of the incubation period, it was shown that these particles retained all the labeled peptide molecules, apparently in bound form. In prolonged experiments the ribosomes synthesized greater quantities of polypeptides than of protein. The synthesizing activity of the whole system was abolished by pretreatment of either ribosomes or supernatant phase with pancreatic ribonuclease. The results suggested that the process of gramicidin and tyrocidine formation resembles that of protein biosynthesis.

In view of the great number and variety of polypeptides found in living cells, the elucidation of the biosynthetic pathways of these substances presents a challenging problem. It is of particular interest to determine whether or not the mechanisms of polypeptide biogenesis resemble the Zamecnik cycle of reactions, which is commonly believed to describe protein synthesis.

The gramicidin and tyrocidine groups of antibiotic peptides found in the Dubos-Hotchkiss strain of *B. brevis* offer a number of attractive features for the above purpose (Okuda *et al.*, 1963). The composition and structures of the cyclic tyrocidines A, B, and C are known (Craig *et al.*, 1949), while the amino acid composition (although not the sequences) of the gramicidin peptides is fairly well worked out (Ramachandran, 1963; Ishii and Witkop, 1963). The latter substances are commonly thought to have closed rings, but this has not been proved.

A previous study (Okuda *et al.*, 1963) was concerned with the biosynthesis of gramicidins and tyrocidines in growing cultures. The present paper describes a cell-free system, prepared from sonicates of *B. brevis*, which is active in incorporating component amino acids into the two groups of polypeptides. The roles of major components of this system, the energy requirements, cofactors, and optimal conditions have all been investigated. In view of the presence of ornithine, certain D-amino acids, and amino acid amides in the peptide molecules, it was of added interest to investigate the modes of utilization of these components in the biosynthetic process.

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EXPERIMENTAL

Isotopic Amino Acids.—The sources and specific activities ($\mu c/\mu m$) were as follows: New England Nuclear Corporation: DL-leucine-1-C^{14}, 17; ethanolamine-2,3-C^{14}, 23; D-valine-U-H^3, 2.0; D-phenylalanine-U-H^3, 4.0. California Corporation for Biochemical Research: DL-valine-1-C^{14}, 9.9; DL-ornithine-2-C^{14}, 1.14; DL-phenylalanine-1-C^{14}, 12. Schwarz Bio-Research, Inc.: L-alanine-1-C^{14}, 78; L-proline-1-C^{14}, 97; L-aspartic acid, 113. Volk Radiochemical Company: L-glutamic acid-U-C^{14}, 2.0. Radiochemical Centre (England): L-isoleucine-U-C^{14}, 6.0; glycine-1-C^{14}, 8.0; DL-tryptophan (indoyl [alanine-3-C^{14}]), 1.0.

The two D-amino acids were originally purchased from the California Corporation for Biochemical Research. They were tested for optical purity with L-amino acid oxidase of snake venom (Sigma Chemical Company). Warburg determinations indicated insignificant quantities of O_2 consumption under conditions which resulted in 100% oxidation of L-phenylalanine and L-valine. Following tritiation by the method of Wilzbach (1959), the preparations were exhaustively freed of labile H^3 by the usual exchange procedures and then rigorously purified by paper chromatography. The final products contained about 0.01% impurity, as judged by zero-time incorporation experiments. Also, automatic scanning of chromatograms indicated the absence of impurities in the final preparations.

Biochemicals.—Crystalline pyruvate kinase enzyme, phospho(enol) pyruvic acid, adenosine triphosphate (ATP), and reduced glutathione were purchased from Sigma Chemical Company. Crystalline pancreatic deoxyribonuclease and ribonuclease were obtained from Worthington Chemical Company. Samples of Dubos-Hotchkiss gramicidin, tyrocidine, and tyrothricin were kindly provided by the Wallerstein Company.